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Role of the H Helix in Heparin Binding to Protein C Inhibitor*

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Protein C inhibitor (PCI) is a plasma serine proteinase inhibitor (serpin) that is a major physiological regulator of activated protein C. Inhibition of its target proteinase is accelerated by heparin in a reaction that involves the binding of both inhibitor and proteinase to heparin to form a ternary complex. This study was undertaken to understand the role of the H helix region (residues 264–278) of PCI in heparin binding and used (i) a recombinant truncated PCI fusion protein of the first 294 residues, (ii) H helix synthetic peptides containing single Arg/Lys → Glu substitutions, and (iii) site-directed Ala mutagenesis of 4 basic residues (Arg-269, Lys-270, Lys-276, and Lys-277) in the H helix region of full-length recombinant PCI (rPCI) expressed in *Baculovirus*. The PCI fusion protein interfered in heparin-accelerated PCI-proteinase inhibition reactions, and it bound to heparin-Sepharose. Compared to the wild-type PCI fusion protein, deletion of the H helix from the fusion protein resulted in a reduction of both heparin-Sepharose binding and the ability to compete for heparin during PCI-proteinase inhibition reactions. Competition assays with H helix synthetic peptides revealed that the R269E altered peptide was the least effective at blocking heparin-catalyzed PCI-proteinase inhibition reactions. Compared with full-length active wild-type rPCI, R269A:K270A and K276A:K277A rPCI both had reduced heparin-Sepharose binding, but only R269A:K270A rPCI showed a loss of heparin-accelerated proteinase inhibition for both activated protein C and thrombin. We conclude that a major heparin-binding site of PCI is the H helix, unlike its heparin-binding serpin homologues antithrombin and heparin cofactor II, which bind heparin primarily through the D helix.

Protein C inhibitor (PCI)[1] is a plasma glycoprotein that is a major physiological regulator of the anticoagulant proteinase activated protein C (APC) (for a review, see Refs. 1 and 2). In addition to activated protein C, PCI can also inhibit thrombin and several other proteinases involved in coagulation, fibrinolysis, and reproduction (3–9). PCI is a member of the serine proteinase inhibitor (serpin) superfamily of proteins (10), of which α_1-proteinase inhibitor is the prototype (11). Like the serpins antithrombin and heparin cofactor II, the reaction of PCI with some of its target proteinases is accelerated by heparin and other glycosaminoglycans (3, 12). Heparin-accelerated thrombin inhibition by these three serpins appears to follow the template model, in which both serpin and proteinase bind to heparin to form a ternary complex (13–18). However, the heparin-induced rate increase is modest for PCI compared with antithrombin and heparin cofactor II (18).

The location of the heparin-binding site in PCI is still unresolved. Heparin-binding proteins are known to contain clusters of basic amino acid residues that form centers of high positive charge density and that form electrostatic interactions with the acidic groups of heparin (19). Three regions in the primary structure of PCI contain clusters of basic amino acids: residues 1–11, 82–90, and 266–278. Based on PCI molecular modeling studies, Kuhn *et al.* (20) proposed that a large positively charged heparin-binding surface is formed by the juxtaposition of the first 15 amino-terminal amino acids (termed the A+ helix)[2] with the H helix region (residues 264–278).[2] A role for the A+ helix in heparin binding was supported by demonstrating that an anti-PCI antibody mapped to the A+ helix blocked heparin-Sepharose binding (20). We provided evidence that the H helix in PCI binds heparin by showing that a H helix synthetic peptide bound to immobilized heparin and interfered with heparin-catalyzed serpin-proteinase inhibition reactions (12). In contrast, an A+ helix synthetic peptide and a peptide containing PCI residues 82–90 were less effective at blocking heparin-catalyzed PCI-proteinase inhibition reactions (12). The work presented here provides more compelling evidence of a role for the H helix in heparin binding to PCI. We have used a recombinant truncated PCI fusion protein expressed in bacteria, altered synthetic peptides, and site-directed mutagenesis of full-length recombinant PCI expressed in *Baculovirus* to examine the H helix of PCI.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were purchased from the oligonucleotide synthesis facility of the Department of Pathology, University of North Carolina (Chapel Hill, NC) or from National Biosciences. Restriction enzymes were from Promega or New England Biolabs Inc. Moloney murine leukemia virus reverse transcriptase, T4 DNA ligase, and mung bean exonuclease were obtained from Life Technologies, Inc. *Taq* DNA polymerase and T4 polynucleotide kinase were from Promega, and T4 DNA polymerase and isopropyl-β-D-thiogalactopyranoside were from Boehringer Mannheim. The Sequenase version 2.0 DNA sequencing kit was from United States Biochemical Corp. Plasmid pMAL-c2, *Escherichia coli* strains TB1 and PR700, amylose resin, and bovine

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[1] The abbreviations used are: PCI, protein C inhibitor; rPCI, recombinant PCI; APC, activated protein C; serpin, serine proteinase inhibitor; PEG, polyethylene glycol; BSA, bovine serum albumin; MBP, maltose-binding protein.

[2] Structural regions designated for PCI and all other serpins described here are based on the elements delineated from the three-dimensional structure of α_1-proteinase inhibitor as reviewed by Huber and Carrell (11).

factor Xa were purchased from New England Biolabs Inc. *E. coli* BW313 was a kind gift of Dr. Elmer M. Price (Department of Pharmacology, University of North Carolina, Chapel Hill). 4-(2-Aminoethyl)benzenesulfonyl fluoride HCl was from Calbiochem, and leupeptin, polyethylene glycol (PEG) 8000, Polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide), and bovine serum albumin (BSA) were from Sigma. The following proteinase chromogenic substrates were used: tosyl-Gly-Pro-Arg-*p*-nitroanilide (Chromozym TH; Boehringer Mannheim) for thrombin and Lys-benzyloxycarbonyl Pro-Arg-*p*-nitroanilide (Spectrozyme PCa; American Diagnostica Inc.) for APC. Human α-thrombin was purified as described previously (21), and human APC was purchased from Haematologic Technologies Inc. Human plasma PCI was purified as described (22) with modifications involving affinity chromatography on an anti-PCI monoclonal antibody column instead of DEAE-Sepharose fractionation. Human plasma antithrombin was purified as described previously (23). Unfractionated heparin was obtained from Diosynth (Oss, The Netherlands). Heparin-Sepharose was purchased from Pharmacia Biotech Inc.

Production of Truncated PCI Fusion Protein—PCI cDNA was obtained by first-strand cDNA synthesis of HepG2 (a hepatoma cell line) total RNA using a random hexameric oligonucleotide primer, followed by polymerase chain reaction amplification with two PCI-specific oligonucleotides that omitted the signal sequence and engineered 5′- and 3′-*Nco*I restriction sites (5′-TCCTCCATGGCTCACCGCCACCACCCCCGGGA-3′ and 5′-TTTTCCATGGGAGAAGCCCCACCTCAGGGGCG-3′). The PCI cDNA was subcloned into phagemid pSL1190 to add 5′-*Eco*RI and 3′-*Bam*HI flanking sequences, which permitted insertion into M13mp19 for mutagenesis. The oligonucleotide-directed mutagenesis method of Kunkel *et al.* (24) was used. Briefly, uracil-rich single-stranded DNA was obtained by infection of *E. coli* strain BW313 with recombinant PCI/M13mp19 phage. The purified DNA was used as a template for *in vitro* annealing and elongation of a mutagenic oligonucleotide (5′-GCTCCTATCAGTGAGAGAAAGTCC-3′) that incorporated a stop codon at position 987–989 (10), corresponding to residue 295 in PCI. *E. coli* strain DH5αF′ was transformed with the heteroduplex DNA. A mutant clone was selected and fully sequenced using the dideoxynucleotide chain termination method (33) (Sequenase version 2.0). This modified cDNA (termed PCI_{294}) was excised from M13mp19 by *Nco*I digestion, treated with mung bean exonuclease to remove the sticky ends, and ligated into the *Xmn*I site of pMAL-c2, a prokaryotic vector that expresses foreign DNA as a fusion protein with *E. coli* maltose-binding protein (MBP). *E. coli* strain TB1 was transformed by electroporation. A clone was selected that contained the PCI_{294} insert in the correct orientation and reading frame and that was missing the amino-terminal codon for alanine that resulted from the engineered *Nco*I site (PCI_{294}/pMAL-2c) (see Fig. 1).

PCI_{294} helix deletion mutants were also generated by using the oligonucleotide-directed mutagenesis method of Kunkel *et al.* (24) on DNA cassettes removed from PCI_{294}/pMAL-2c and inserted into M13mp19, a *Sac*I-*Kpn*I DNA fragment for the A+ helix deletion, and a *Kpn*I-*Bam*HI fragment for the H helix deletion. Coding sequence for the A+ helix (amino acids 1–15 of the mature protein) was deleted using a 30-mer composed of the 15 nucleotides 5′ and 3′ to the 45 bases being deleted (5′-GGGATCGAGGGAAGGCATGTAGGTGCCACG-3′). Coding sequence for PCI amino acid residues 264–277 (H helix region) was deleted using a similarly designed oligonucleotide (5′-GTGGAGAATGGACTGAGGCAGCTGAGCTT -3′). Mutated cassettes were fully sequenced and subcloned back into the PCI_{294}/pMAL-2c expression vector to replace the corresponding wild-type sequence. The ΔA+ helix/ΔH helix double mutant was constructed by replacing the wild-type *Sac*I-*Kpn*I cassette in ΔH-PCI_{294}/pMAL-2c with the mutant ΔA+skPCI_{294} cassette.

For large-scale fusion protein production, the expression vector constructs were transformed into *E. coli* strain PR700. Fusion protein was purified from cell extracts by amylose resin affinity chromatography according to the manufacturer's procedure. The purified fusion proteins were dialyzed into 20 mM HEPES, 10 mM NaCl, 0.1% PEG, 1 mM NaN_3, pH 7.4, and their concentrations were determined by the dye binding assay of Bradford (32) using BSA as the standard. Immunoblots showed that the wild-type and ΔH helix fusion proteins were detected by a monoclonal antibody to the A+ helix of PCI, while the ΔA+ helix and ΔA+ helix/ΔH helix fusion proteins were not (data not shown).

Baculovirus Expression of Full-length rPCI—Wild-type rPCI was generated and characterized as described previously (25). H helix substitution mutants were generated as follows. A 1-kilobase DNA cassette containing the 3′-end of PCI cDNA followed by 500 base pairs of the transfer vector pVL1393 downstream of the polylinker was excised from PCI/pVL1393 by limited *Kpn*I digestion and subcloned into M13mp19 for mutagenesis using the method of Kunkel *et al.* (24). Oligonucleotide 5′-GAGAAAACGCTGGCGGCGTGGCTTAAGATG-3′ was employed to replace Arg-269 and Lys-270 with alanines (R269A:K270A rPCI), and oligonucleotide 5′-CTTAAGATGTTCGCAGCGAGGCAGCTCGAG-3′ was used to replace Lys-276 and Lys-277 with alanines (K276A:K277A rPCI). At the same time, the internal *Kpn*I site from the vector polylinker was modified (made uncuttable) with oligonucleotide 5′-GGATCCCGGGTATATTCTGAATTCC-3′ to facilitate cloning back into the PCI/pVL1393 transfer vector construct. Mutant cassettes were sequenced and subcloned back into PCI/pVL1393 to replace wild-type DNA, and the transfer vector was sequenced again to confirm the presence of the desired substitutions prior to homologous recombination. Generation of high titer recombinant viral stocks and infection of High-Five™ cells (Invitrogen) were performed as described previously (25). Wild-type rPCI was purified by batch absorption to heparin-Sepharose as detailed (25). H helix mutants were purified with the following modifications. Conditioned medium was diluted 1:2 with 20 mM HEPES, 10 mM NaCl, 0.1% PEG, 0.02% NaN_3, pH 6.5, to lower the ionic strength before batch absorption to heparin-Sepharose. The resin was washed twice with this HEPES buffer, pH 6.5, containing 150 mM NaCl and once with this buffer containing 250 mM NaCl before elution with 1 M NaCl in HNPN buffer (20 mM HEPES, 150 mM NaCl, 0.1% PEG, 0.02% NaN_3, pH 7.4). The proteins were dialyzed into HNPN buffer and quantitated by enzyme-linked immunosorbent assay and thrombin inhibition rate constants in the absence of heparin. Typical protein yields from 50 ml of culture medium (from two T-150 flasks of confluent High-Five™ cells) were 70 µg of wild-type rPCI and 15–40 µg of the H helix mutants.

Competition Assays—Fusion protein competition in proteinase inhibition assays was performed at ambient temperature in 96-well microtiter plates under pseudo first-order conditions. Increasing concentrations (0.05–5 µM) of fusion protein or MBP alone were added to assay mixtures containing inhibitor, 1 µg/ml heparin, and 2 mg/ml BSA in HNPN buffer. Reactions were started by the addition of proteinase. Inhibitor-proteinase concentrations used were 50 nM PCI and 5 nM thrombin, 100 nM PCI and 10 nM APC, and 10 nM antithrombin and 1 nM thrombin. After incubation, remaining proteinase activity was measured by hydrolysis of 0.15 mM chromogenic substrate with 2 mg/ml Polybrene. Second-order proteinase inhibition rate constants (k_2) were calculated as $-\ln(a)/t[I]$, where a is the fractional proteinase activity remaining relative to the uninhibited control, t is the incubation time, and [I] is the inhibitor concentration. Fig. 2 plots relative proteinase activity (a) as a function of increasing fusion protein concentrations. Because relative thrombin activity in the presence of inhibitor but in the absence of fusion protein (*i.e.* no fusion protein control, *min a*) ranged from 0.15 to 0.3 in multiple assays, the data were normalized by fixing the *min a* value at 0.2 as follows: normalized a = 0.2 + 0.8(old a − old *min a*)/(1.0 − old *min a*). Control experiments verified that fusion proteins did not inhibit proteinase chromogenic activity and did not affect proteinase inhibition in the absence of heparin.

PCI H helix synthetic peptides for competition assays were assembled by CDI (Lelystad, The Netherlands). Purity was >85% by high pressure liquid chromatography. Peptides (0.1–500 µM) were added to assay mixtures containing 40 nM PCI (with APC) or 5 nM PCI (with thrombin), 0.5% BSA, and 10 µg/ml heparin in 50 mM Tris-HCl, 125 mM NaCl, 0.1% PEG 6000, 2 mM $CaCl_2$, pH 7.4. Reactions were performed at 25 °C and started with the addition of 4 nM APC or 100 pM thrombin. After incubation, the remaining chromogenic activity was measured by adding 0.5 mM S-2366 to the assay mixture. The rate of *p*-nitroaniline formation was linearly related to the free proteinase concentration. Control experiments verified that none of the peptides affected proteinase chromogenic activity or the ability of PCI to inhibit APC or thrombin in the absence of heparin.

Heparin-Sepharose Affinity Chromatography—The relative affinity of recombinant and plasma PCIs for immobilized heparin was determined using the fast protein liquid chromatography system of Pharmacia Biotech Inc. and a 1-ml heparin-Sepharose column. Samples were loaded in 20 mM HEPES, 10 mM NaCl, 0.1% PEG, pH 7.4, and eluted with a linear 1.5 ml/min salt gradient of 10 mM to 1.2 M NaCl. 120 µg of the fusion proteins were loaded, and elution was monitored by continuous absorbance at 280 nm readings. 0.5 µg of full-length rPCI was loaded, 30 × 1-ml fractions were collected, and the fractions were analyzed by enzyme-linked immunosorbent assay. The salt gradient and elution profile were plotted, and the salt concentration at which peak elution occurred was calculated. The mean of five to seven runs on two separate columns is reported for the fusion proteins, and the mean of three runs is reported for full-length rPCI and plasma PCI.

Heparin-accelerated Proteinase Inhibition by Full-length rPCI and Mutants—Inhibition assays were performed at ambient temperature in BSA-coated 96-well microtiter plates and contained 5 nM rPCI and 0.5

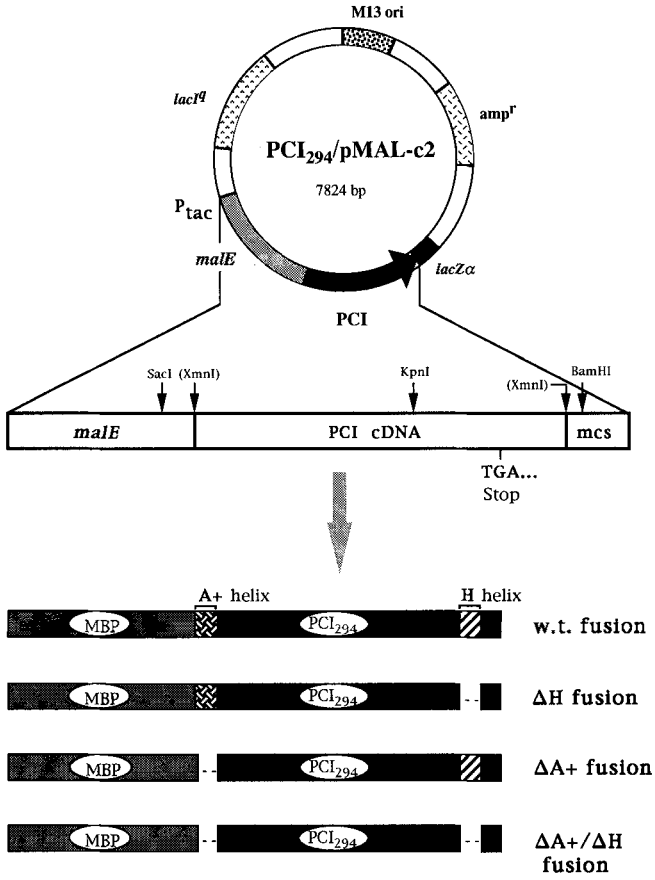

FIG. 1. **Cloning and mutagenesis of rPCI-(1–294) fusion proteins in *E. coli*.** PCI cDNA was modified by the oligonucleotide-directed mutagenesis method of Kunkel *et al.* (24) to incorporate a stop codon at nucleotide 986, corresponding to amino acid 295 in PCI. The cDNA was inserted into pMAL-c2 at the *Xmn*I site to create PCI_{294}/pMAL-c2. A+ helix and H helix deletion fusion mutants were generated from the parent expression construct by mutagenesis of DNA cassettes removed from PCI_{294}/pMAL-c2 as described under "Experimental Procedures." Fusion proteins were expressed in *E. coli* and purified by affinity chromatography on amylose resin. *mcs*, multiple cloning site; *bp*, base pairs; *w.t.*, wild-type.

nM thrombin (or 10 nM rPCI and 1 nM APC), 0.1 mg/ml Polybrene or various concentrations of heparin, and 2 mg/ml BSA in HNPN buffer. Reactions were started with the addition of proteinase, and after incubation, the remaining proteinase activity was measured with 0.15 mM chromogenic substrate containing 2 mg/ml Polybrene. Second-order inhibition rate constants were calculated as described above. Each assay consisted of triplicate determinations, and the mean inhibition rate from three separate assays is reported. For each mutant, preparations from two separate clones were analyzed and gave comparable results.

RESULTS

Recombinant PCI-(1–294) Fusion Proteins—A recombinant PCI fragment with both A+ and H helices but lacking the reactive-site loop region (therefore possessing no inhibitory activity) was expressed in *E. coli* as a fusion protein with MBP and is designated "wild-type" PCI-(1–294) fusion protein. Three helix deletion mutants were also constructed in which residues 1–15 of PCI (A+ helix), residues 264–277 (H helix), or both the A+ and H helices were deleted, and they are designated ΔA+ helix, ΔH helix, and ΔA+ helix/ΔH helix fusion proteins, respectively (Fig. 1). To measure the effect of helix deletions on heparin binding by PCI-(1–294) fusion protein, increasing concentrations of fusion protein were added to an assay system containing thrombin, plasma-derived PCI, and heparin. A heparin-binding protein would be expected to compete with plasma PCI for heparin, thereby decreasing the rate of proteinase inhibition. Wild-type PCI-(1–294) fusion protein blocked heparin-accelerated thrombin inhibition in a dose-dependent manner (IC_{50} = 1 μM) (Fig. 2). Free MBP alone or wild-type fusion protein in the absence of heparin did not have this property (data not shown). The ΔA+ helix fusion protein had a dose-response curve much like that of the wild-type fusion protein, but both the ΔH helix and ΔA+ helix/ΔH helix fusion proteins had a drastically reduced ability to compete with plasma PCI for heparin binding (Fig. 2).

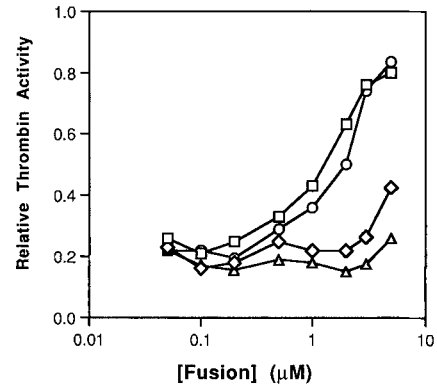

FIG. 2. **Effect of helix deletions on fusion protein competition in heparin-accelerated thrombin inhibition.** Increasing concentrations of wild-type fusion protein (□) or ΔH helix (◇), ΔA+ helix (○), or ΔA+ helix/ΔH helix (△) deletion fusion proteins were added to thrombin inhibition assays containing 50 nM PCI, 5 nM thrombin, and 1 μg/ml heparin. After incubation, the remaining thrombin activity was measured by tosyl-Gly-Pro-Arg-*p*-nitroanilide hydrolysis. The *y axis* shows thrombin activity relative to the activity in the absence of PCI. *Increased thrombin activity indicates decreased inhibition due to competition between PCI and fusion protein for heparin binding.* The mean of normalized data from three separate experiments is presented.

The fusion proteins were also assayed for their effect on heparin-accelerated APC inhibition by PCI and thrombin inhibition by antithrombin. For both inhibitor-proteinase pairs tested at a fixed concentration of 3 μM fusion protein and 1 μg/ml heparin (k_2 = 18.9 × 10^4 M^{-1} min^{-1} for PCI-APC and 18.4 × 10^8 M^{-1} min^{-1} for antithrombin-thrombin in the absence of fusion proteins), the addition of the wild-type or ΔA+ helix fusion protein reduced the rate of proteinase inhibition by both serpins to ~9 or ~16%, respectively, of the inhibition rate in the absence of fusion protein. In contrast, the ΔH helix fusion protein competed less effectively, reducing the rate of heparin-accelerated proteinase inhibition by both serpins to ~53% of the rate without fusion protein present. The wild-type fusion protein and all three helix deletion fusion proteins also bound to heparin-Sepharose. In contrast, free MBP did not bind and was recovered in the flow-through fraction. The sodium chloride concentration required for peak elution was determined to provide a measure of relative affinity. The fusion proteins bound to heparin-Sepharose with highest to lowest relative affinity in the following order: wild-type (590 mM NaCl) > ΔA+ helix (500 mM NaCl) > ΔH helix (410 mM NaCl) > ΔA+ helix/ΔH helix (260 mM NaCl).

Altered PCI H Helix Synthetic Peptides—The PCI fusion protein study directed our focus to the H helix of PCI. To test if certain basic residues in the H helix may play a more prominent role than others in the PCI-heparin interaction, synthetic peptides were assembled corresponding to PCI-(264–283) (SE**K**TL**RK**WL**K**MF**KKR**QLELY) in which each Lys and Arg was substituted individually with Glu. The heparin binding properties of the peptides were assessed in competition assays and shown to decrease the rate of proteinase inhibition in a dose-dependent manner (Fig. 3*A*), and they generated sigmoidal dose-response curves like those obtained for the PCI fusion proteins (data not shown). All the altered peptides were less

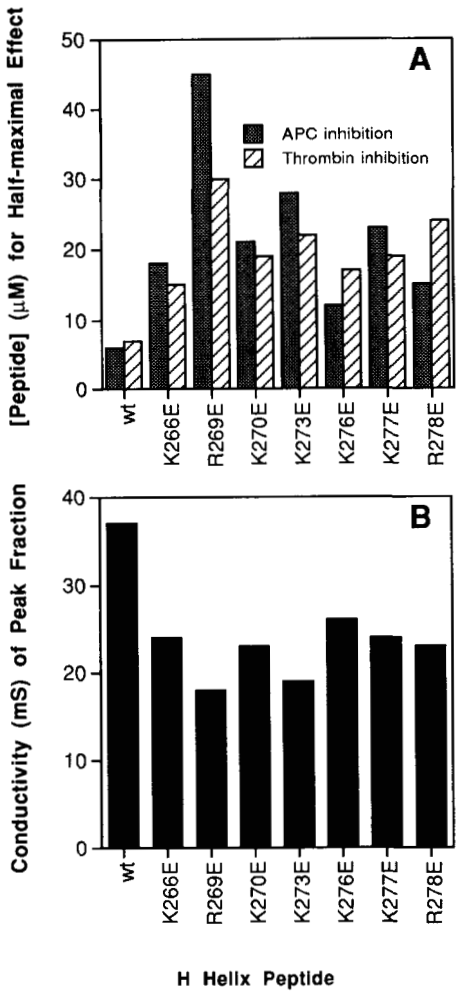

FIG. 3. **Effect of altered PCI H helix peptides on interaction with heparin.** *A*, increasing concentrations of the wild-type (*wt*) H helix peptide (PCI-(264–283), SE**K**TL**RK**WL**K**MF**KKRQ**LELY) and seven altered peptides, in which each Lys and Arg was individually substituted with Glu, were tested for their ability to interfere in heparin-accelerated PCI-thrombin and PCI-APC inhibition assays as described under "Experimental Procedures." The peptide concentrations required for 50% reduction (IC_{50}) of the inhibition rate constant are given on the *y axis*. The mean of three separate APC experiments and two thrombin experiments is reported. *B*, peptides were loaded onto a heparin-Sepharose column (2-ml bed volume) equilibrated in 50 mM phosphate buffer, pH 7.4. They were eluted with a 20-ml gradient of 0–750 mM NaCl in phosphate buffer at 0.5 ml/min and at room temperature with detection at 214 nm. Conductivity (microsiemens (*mS*)) measurements were made for the peak elution fraction, and the mean of two to eight separate runs for each peptide is shown.

competitive, requiring higher concentrations than the wild-type H helix peptide (IC_{50} = 5 μM) to get the same degree of interference (Fig. 3*A*). In particular, H helix peptide R269E was the least competitive for both APC (IC_{50} = 45 μM) and thrombin (IC_{50} = 30 μM) inhibition by PCI-heparin. Furthermore, a similar trend was seen with the altered H helix peptides when assessed for heparin-Sepharose binding (Fig. 3*B*).

Site-directed Mutagenesis of H Helix Basic Residues in Full-length rPCI—The role of specific basic amino acids in the H helix region of PCI was further assessed using full-length active rPCI produced by a *Baculovirus* expression system. Two H helix double mutants were generated by replacing Arg-269 and Lys-270 with Ala (R269A:K270A rPCI) and Lys-276 and Lys-277 with Ala (K276A:K277A rPCI). The H helix mutants were compared with wild-type rPCI for their heparin binding properties. Wild-type rPCI eluted from heparin-Sepharose at 630 mM NaCl. Both R269A:R270A and K276A:K277A rPCI showed reduced relative heparin-Sepharose binding, both eluting at 460 mM NaCl (Table I).

Second-order rate constants for APC and thrombin inhibition by rPCIs were determined in the absence and presence of increasing concentrations of heparin. Wild-type rPCI and both H helix substitution mutants yielded typical bell-shaped heparin template curves (Fig. 4). APC inhibition in the absence of heparin yielded rate constants of 4.71×10^4, 4.80×10^4, and 3.90×10^4 M^{-1} min^{-1} for wild-type, R269A:K270A, and K276A:K277A rPCI, respectively. In the presence of optimal heparin concentrations, APC inhibition rates for wild-type, R269A:K270A, and K276A:K277A rPCI were accelerated 32-, 15-, and 39-fold, respectively (Fig. 4 and Table I). In the absence of heparin, thrombin inhibition rates for wild-type, R269A:K270A, and K276A:K277A rPCI were 1.11×10^6, 0.97×10^6, and 0.79×10^6 M^{-1} min^{-1}, respectively. In the presence of optimal heparin concentrations, thrombin inhibition rates for wild-type, R269A:K270A, and K276A:K277A rPCI were stimulated 27-, 11-, and 32-fold, respectively (Fig. 4 and Table I). For both APC and thrombin inhibition, R269A:R270A and K276A:K277A rPCI required more heparin to reach the maximal inhibition rate than did wild-type rPCI.

DISCUSSION

We utilized three approaches to assess the role of the H helix as a putative heparin-binding site in PCI. A recombinant truncated PCI fusion protein was shown to interfere in heparin-accelerated proteinase inhibition and to bind heparin-Sepharose. Deletion mutagenesis showed that the H helix played a greater role than the A+ helix in heparin binding by the PCI fusion protein and supports the hypothesis that the H helix is an important heparin-binding site in PCI. PCI H helix peptides were also shown to bind heparin in competition assays. Substitution of specific basic residues with Glu reduced heparin binding by the peptides compared with the wild-type peptide. The peptide study further implies the importance of electrostatic interactions in PCI-heparin binding and indicates in particular that Arg-269 may be especially important. These results refine and extend previous reports that implicated the H helix as a putative heparin-binding site in PCI.

The 7 basic residues in the H helix region can be grouped into two clusters that have homology to two heparin-binding consensus sequences (19), and Arg-269 is in the first cluster. The peptide data do not provide compelling evidence for the importance of one basic cluster or consensus sequence over the other. To address that question, we generated full-length active rPCI and two H helix double substitution mutants corresponding to adjacent basic residues in each cluster, R269A:K270A and K276A:K277A rPCI. Arginine 278 was not mutated in rPCI because (*a*) a basic residue is conserved in that position in many serpins (11), (*b*) Arg-278 is at the boundary of the next exon, and (*c*) an α-helical wheel plot of PCI-(264–283) predicts that Arg-278 lies on the opposite face of a helix to the other basic residues (12). In heparin-accelerated APC and thrombin inhibition assays, R269A:K270A and K276A:K277A rPCI required more heparin to reach the maximal inhibition rate compared with wild-type rPCI, consistent with their reduced relative affinity for heparin-Sepharose. However, K276A:K277A rPCI achieved essentially the same maximal heparin acceleration as wild-type rPCI, whereas R269A:K270A rPCI exhibited a markedly reduced heparin stimulation. These results indicate that while both Arg-269 and/or Lys-270 and Lys-276 and/or Lys-277 appear to contribute to overall heparin binding by PCI, only Arg-269 and/or Lys-270 is required for the maximal heparin-accelerated proteinase inhibition response. These full-length rPCI mutants suggest that reduced heparin affinity does not necessarily result in reduced heparin-accelerated proteinase inhibi-

TABLE I
Heparin-accelerated proteinase inhibition by rPCI and H helix mutants

rPCI	Heparin-Sepharose affinity[a]	APC inhibition			Thrombin inhibition		
		Heparin Optimum[b]	Maximum rate	Rate increase[c]	Heparin optimum	Maximum rate	Rate increase
	mM NaCl	*μg/ml*	$k_2 \times 10^{-6}$ *M⁻¹ min⁻¹*	*-fold*	*μg/ml*	$k_2 \times 10^{-7}$ *M⁻¹ min⁻¹*	*-fold*
Wild-type	630 ± 0	10	1.52	32	5	3.02	27
R269A:K270A	460 ± 50	100	0.73	15	50	1.05	11
K276A:K277A	460 ± 50	50	1.50	39	10	2.53	32

[a] Relative heparin-Sepharose affinity is given as the NaCl concentration required for peak elution (n = 3).
[b] The heparin optimum is the concentration at which the maximum inhibition rate occurs.
[c] The rate increase is calculated as the ratio of the maximum rate to the rate in the absence of heparin.

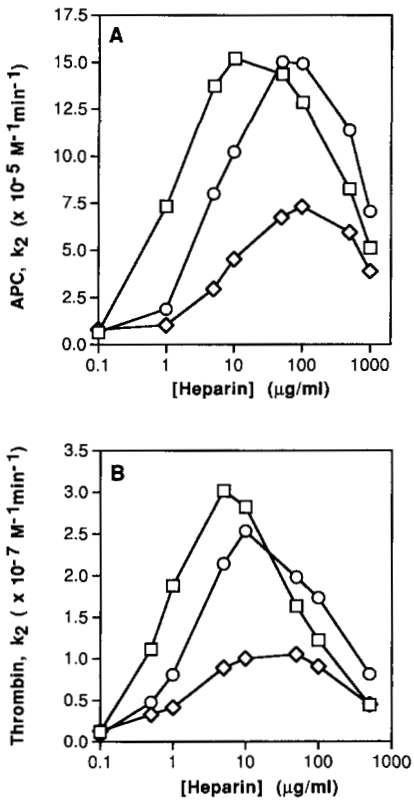

FIG. 4. **Heparin-accelerated proteinase inhibition by rPCI and H helix mutants.** The rate of APC (*A*) and thrombin (*B*) inhibition by wild-type rPCI (□) and by rPCI H helix substitution mutants R269A:K270A (◇) and K276A:K277A (○) was measured as a function of heparin concentration as described under "Experimental Procedures." The mean inhibition rate constants (k_2) from three separate APC experiments and four separate thrombin experiments are reported.

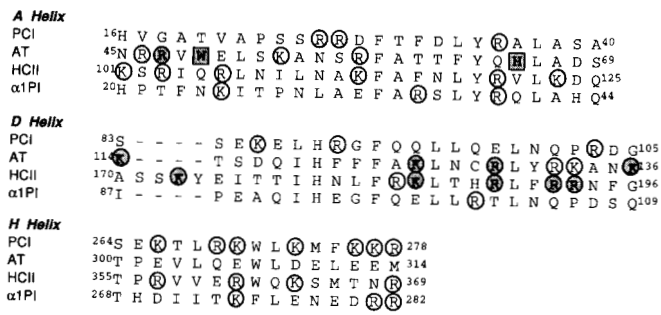

FIG. 5. **Amino acid sequence alignment of the putative heparin-binding sites in heparin-binding serpins.** Selected sequences of PCI, antithrombin (*AT*), and heparin cofactor II (*HCII*) are aligned with the sequence of α_1-proteinase inhibitor (*α1PI*), a non-heparin-binding serpin, as described by Huber and Carrell (11). Basic residues are *circled* for emphasis. Residues implicated in heparin binding by antithrombin (reviewed in Refs. 26 and 27) and in heparin/dermatan sulfate binding by heparin cofactor II (28–31) are *shaded*.

tion. PCI binding to heparin may include random association events, not just the productive binding of serpin to heparin that catalyzes proteinase inhibition. Therefore, it is possible that some basic residues are required for the "optimal alignment" of PCI on the heparin molecule, while others only contribute to the overall "tightness" of binding.

A role for the H helix in heparin binding to PCI is in sharp contrast to that in antithrombin and heparin cofactor II. Using a combination of chemical modification studies, analysis of natural human variants, and site-directed mutagenesis, the heparin-binding site in antithrombin and heparin cofactor II has been localized primarily to the D helix, with additional contributions by residues in the A helix for antithrombin (26–31). The conserved distribution of basic residues in the D helices of antithrombin and heparin cofactor II is conspicuously absent in PCI (Fig. 5). PCI's C1 and D helices collectively contain 4 basic residues, but a synthetic peptide corresponding to this region (residues 80–93) did not readily bind to heparin (12). The basic charge density in the H helix of PCI is greater than that in antithrombin, heparin cofactor II, and α_1-proteinase inhibitor (Fig. 5). Interestingly, acidic residues in antithrombin align with most of the basic residues in the H helix of PCI.

The location of PCI's primary heparin-binding site in a position different from the heparin-binding sites in antithrombin and heparin cofactor II may help explain PCI's more modest response to heparin. Under identical thrombin inhibition assay conditions, optimal heparin concentrations elicited a 9000-fold rate increase for heparin cofactor II, a 2500-fold rate increase for antithrombin, but only a 15-fold rate enhancement for PCI (18). This difference in rate enhancement does not correlate with serpin-heparin affinity (18). Since heparin acts as a template to bind both serpin and proteinase, the "orientation" of the serpin reactive site and the proteinase active site could be governed, at least in part, by how the serpin binds to heparin. Binding heparin through the H helix instead of the D helix may alter the reaction geometry such that PCI is put in a less favorable orientation to react with heparin-bound proteinase.

In summary, we have shown that the H helix is an essential component of the heparin-binding site in PCI. It remains to be seen what contribution other structural regions, such as the A+, C1, and D helices, or other basic residues in the H helix make to both heparin binding and heparin-accelerated proteinase inhibition by PCI.

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